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Abstract

In many insect species, sperm-depleted males (SDMs, i.e. males that have exhausted their sperm after a given number of matings) remain sexually active, and continue to mate females. Here we investigated the behavioral modifications that occur in both sexes of the parasitoid *Asobara tabida* Nees (Hymenoptera: Braconidae), after matings by fertile males and sperm-depleted males. We show that (i) virgin females, mated females, and females mated to a SDM exhibited different behaviors, and that (ii) males responded differently to females depending on whether the females had previously mated with an SDM or not. Our findings demonstrate that SDM influenced the reproductive behavior of both males and females, especially with regard to male responsiveness and female attractiveness. These findings are discussed in the context of adaptive behavior and fitness maximization in both males and females.

Keywords

Sperm-depleted male, hymenopteran parasitoids, mate choice, *Asobara tabida*, conspecific behavior

Introduction

Sperm is not an infinite and free-cost resource to produce. In polygynous species, mating several females successively in a short period of time may result in sperm depletion: The sperm supply can be drained after a given number of females mated (Wedell et al. 2002). Sperm depletion can be temporary (in synspermatogenic species) or definitive (in prospermatogenic species), depending on the ability of males to produce new sperm during their adult life (Damiens and Boivin 2006; Boivin 2013). Sperm-depleted males (SDMs) have been found in many taxa, such as mammals (Preston et al. 2001), birds (Pizzari et al. 2003), lizards (Olsson et al. 2004), crustaceae (Rubolini et al. 2007) and fish (Smith et al. 2009). In insects, sperm depletion occurs in many species and SDMs individuals remain sexually active as they are still able to attract receptive females and to mate them (Simmonds 1953; Laing and Caltagirone 1969; Gordh and DeBach 1976; Assem et al. 1979; Nadel and Luck 1985; Ramadan et al. 1991; Ode et al. 1995; Damiens and Boivin 2005; Rönn et al. 2008; Steiner et al. 2008). It was suggested that continuing to mate is an adaptive strategy of SDMs to decrease the fitness of fertile males (Damiens and Boivin 2006). The authors indeed demonstrated that *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae) females have a decreased ability to store sperm after being mated by SDMs (Damiens and Boivin 2006). To continue to mate is thus viewed as a SDMs' strategy to increase their relative fitness comparing to this of other males. On the other hand, such behavior may persist over generations because of the lack of selective pressure counter-selecting it (SDMs can obviously not produce progeny anymore). Whatever the adaptive significance of the SDM mating behavior, its effect on the behaviors of conspecifics (both males and females) remains largely unknown.

Females' behaviors may be modified after copulation; after mating, females usually become refractory to subsequent copulations for a given time, even without sperm transfer

43 (Gillott 2003). This refractoriness to mating is caused by chemicals contained in the seminal
44 fluids transferred during mating or by the male's behavior (Alcock 1994; Gillott 2003; Wedell
45 2005; Yamane 2013). The transfer of chemicals to the mated female is therefore viewed as a
46 male strategy to suppress the subsequent reproductive activity of females, thus decreasing the
47 likelihood of sperm competition (Ejima et al. 2007). For example, Radhakrishnan et al. (2009)
48 showed that the transfer of seminal fluid in *Bactrocera tryoni* Froggatt (Diptera: Tephritidae)
49 during mating induces female sexual inhibition. In this case, the behavior of the mated
50 females is modified by SDM resulting in fewer opportunities for other males to access them.
51 In many species, females mated to a SDM become less receptive to mating than virgin
52 females, suggesting that these females no longer invest in pheromone biosynthesis after
53 mating (Steiner et al. 2008). We however note that in at least another species, females mated
54 to a SDM remain sexually active (Damiens and Boivin 2006).

55 Male mating may also modify the behavior of conspecific males. Males are known to
56 be choosy just like females (Wedell et al. 2002), and the females' mating status influences
57 male choice. For example, the male preferences for virgin females and/or for females that
58 they have never previously encountered have been observed in many insects species
59 (Bonduriansky 2001; Wedell et al. 2002; Ferveur 2005; Bateman and Fleming 2006;
60 Everaerts et al. 2010). Sexual pheromones transferred by males during mating can mark
61 females as mated, making them less attractive to other males (Findlay et al. 2008). In
62 *Drosophila*, mating induces changes in the female cuticular profile, which reduces the time
63 spent in copulation by subsequent males (Friberg 2006; Everaerts et al. 2010). Previous
64 mating can therefore influence the choice of other males, which generally prefer to mate with
65 virgin females.

66 Because all the behavioral modifications detailed above do not require sperm transfer,
67 such modifications are likely to occur after mating by a SDM. The aim of our study was thus

to investigate the behavioral consequences of SDMs mating behavior in both sexes. In this system, conflict between the sexes arises, as females mated to a SDM are still unfertilized and so would gain by remaining attractive to other males in order to ensure egg fertilization. On the other hand, SDMs would gain by decreasing the attractiveness of the females they mated in order to reduce other males' opportunity to access them. Given that, we hypothesize that (i) females mated to a SDMs should be as receptive as virgin females in order to get access to further insemination, (ii) males should be more attracted to virgin females than females mated to a SDM and mated females.

To test these hypotheses, we used the parasitoid *Asobara tabida* Nees (Hymenoptera: Braconidae), a haplodiploid species in which sons result from unfertilized eggs while daughters result from fertilized eggs, allowing us to determine if the females had been successfully inseminated from the resulting sex ratio obtained – the presence of at least one daughter in the progeny is a proof of insemination. *Asobara tabida* males exhibit a stereotyped courtship behavior consisting of wing fanning, antennation, head movements, and leg tapping (Matthews 1975; Carton et al. 1986; Dufour et al. 2012). No postcopulatory behavior is reported for this species (Stökl et al. 2014). *Asobara tabida* males are generally sperm-depleted after a large number of matings (about 13 matings), but continue to mate after sperm depletion (Louâpre, Dufour, personal observation). Chemical communication is of great importance for mating as *A. tabida* males respond to chemicals deposited by females and increase their residence time in a patch previously exposed to females (Dufour et al. 2012). More recently, Stökl et al. (2014) highlighted the remarkable diversity of the sex pheromones of males and females in *A. tabida*. They showed that mating behavior in this species is regulated by a complex combination of chemicals rather than by a single molecule.

Materials and Methods

Insect rearing and general methods

The *Asobara tabida* strain used in this study was collected in Chasse-Sur-Rhône (France, 45°34'N, 4°48'E) in September 2008, and reared in the laboratory on second-instar *Drosophila subobscura* hosts in a controlled-climate room (20°C, 60% relative humidity, and 16:8 h Light:Dark). A few days before emergence, parasitized pupae from the culture were isolated in gelatin capsules (0.68 mL) to obtain virgin parasitoids of known age. Freshly emerged males and females were kept separately in plastic tubes (h = 10 cm, Ø = 2.5 mm) and fed with diluted honey solution.

Choice tests

Experiments were performed in the controlled-climate room used for the rearing. Males were 1-2 days old and females were an equal mixt of 1-4 days old (the two females used in each if the choice test were of comparable ages in order to avoid potential confounding effect of age). A light table was used to maintain the individuals on the bottom of a Petri dish (Ø = 90 mm) by phototaxis. All experiments were video-taped for a period of 15 minutes, and analyzed using the computer software package The Observer v3.0 (Noldus, Wageningen, The Netherlands). During each choice test, we noted (i) the male behavior (courtship, contact with the female, mating) (ii) the identity of the female toward which the male directed its behavior, and (iii) the behavior of the female toward the male (i.e. approaches and escapes, acceptance or rejection). The identity of the female toward which the first courtship was directed was also noted as a proxy of the male preference before any reaction from the female. Mating is indeed the result of both active and apparent choices of the two sexes and the first courtship give supplementary information compared to the mating with a given female (Martel and Boivin 2011).

Experiment 1: Female mated to a SDM vs. *virgin female*

In this experiment, a virgin male was placed in the presence of both a female mated to a SDM and a virgin female in order to find out whether the male would perceive them as different resources. To obtain a female mated to a SDM, a single male mated a sequence of 30 virgin females over a period of 6 hours (from rank-1 to rank-30 females). Although *Asobara tabida* males become sperm-depleted after about 13 matings (Louâpre, Dufour, personal communication), we used females from rank-24 to rank-29 and we isolated the rank-23 and rank-30 females in a plastic tube containing a substrate of agar-nipagine and 20 second-instar *D. subobscura* larvae. The sex-ratio produced was noted, and only data from true SDMs and females mated to an actual SDM – whose offspring had a sex-ratio of 1 (i.e. male-only progeny) – were used for the analysis. In the rearing conditions, inseminated females indeed lay at least several females in a dozen of hosts, so that a male-only progeny obtained in 20 hosts is likely to be a strong clue for an absence of fertilization of the females. One female mated to a SDM (only females mated to different SDMs were used for each of the repetitions) and virgin female were then placed in the same clean Petri dish. A virgin male was introduced at the beginning of the experiment and placed equidistant from the two females. Thirteen dual choice tests were performed.

Experiment 2: Female mated to the same SDM vs. virgin female

In this experiment, each SDM was placed in the presence of a female he had previously mated without sperm transfer (randomly selected from rank-24 to rank 29 in the sequence of copulations) and a virgin female. Both the female mated to the SDM and the virgin female were placed in the same clean Petri dish. The SDM was introduced at the beginning of the experiment and placed equidistant from the two females. Eleven dual choice tests were performed.

Experiment 3: Female mated to the same fertile male vs. *female mated to another fertile male*

In this experiment, a fertile male was placed in the presence of a female he had previously mated and a female that had been mated by another male to determine whether males recognize a female they have previously mated. Ten minutes before the choice test, two virgin males each mated a virgin female in separate plastic tubes (h = 10 cm, Ø = 2.5 mm). The two mated females were then placed in the same clean Petri dish. One of the two males used was introduced at the beginning of the experiment and placed equidistant from the two females. Twenty-two dual choice tests were performed.

Statistical analyses

Statistical analyses were performed using R 2.15.0 software at the critical level $\alpha = 0.05$. The male choices were compared to a 50:50 probability (i.e. no preferential choice) using an exact binomial test. The mean numbers of the types of behavior exhibited by the males and females were analyzed using Wilcoxon tests. Because both virgin females and females mated to a SDMs were used in the first two experiments, the occurrence of approaches and escapes during these two experiments were analyzed by GLM-Poisson procedure, using the mating status of the females (i.e. virgin, pseudo-virgin, or mated) and of the males (fertile or sperm-depleted) as factors. Multiple comparisons were then performed using the glht function of R (multcomp package).

Results

Experiment 1: female mated to a SDM vs. virgin female (fig. 1)

The two types of females exhibited the same number of approaches and escapes toward the virgin male (respectively W = 82, P = 0.915 and W = 78, P = 0.753) (fig. 1.b). Virgin males did not direct their first courtship or their first mating toward one female type rather than the

other (Binomial test, respectively P = 0.581 and P = 0.179) (fig. 1.a). During the course of the experiment, the males exhibited on average the same number of courtships and contacts with females mated to a SDM and virgin females (respectively, W = 68, P = 0.401 and W = 64, P = 0.288) (fig. 1.c). They tended to mate the originally virgin females more frequently than the SDM-mated females during the course of the experiment, but this difference was not significant probably due to the weak number of repetitions (W = 52.5, P = 0.055) (fig. 1.c).

Experiment 2: Female mated to the same SDM vs. virgin female (fig. 2)

Virgin females exhibited more approaches toward the SDM than the females previously mated by him (W = 26.5, P = 0.026), while the number of escapes from the SDM did not differ between both groups of females (W = 46, P = 0.352) (fig. 2.b). The first courtship was not significantly exhibited by SDMs toward one type of females over the other (Binomial test, P = 0.227) (fig. 2.a). On average SDMs directed a similar number of courtships toward females mated by them and virgin females (W = 35, P = 0.401) (fig. 2.c). However, they exhibited more contacts with virgin females than with females they previously mated (W = 29, P = 0.037). They mated the virgin females before the females they previously mated (Binomial test, P = 0.012; fig. 2.a), and mated the originally virgin females more frequently than the SDM-mated females during the course of the experiment, even though she becomes mated after the first mating (W = 13.5, P < 0.001; fig. 2.c).

Experiment 3: Female previously mated by the same fertile male vs. *female previously mated by another fertile male (fig. 3)*

The two types of females exhibited the same number of approaches and escapes toward the fertile male (W = 299, P = 0.104 and W = 199, P = 0.313, respectively) (fig. 3.b). Although fertile males directed their first courtship toward females previously mated by another male more often than toward the ones they had mated themselves (Binomial test, P = 0.004) (fig.

3.a), they exhibited overall the same number of courtships and contacts toward both females (W = 184.5, P = 0.171 and W = 211, P = 0.458, respectively) (fig. 3.c). No mating was observed during this experiment.

Female behavior across the experiments (fig. 1.b and 2.b)

The behavior of virgin females and females mated to a SDM was compared between experiments 1 and 2. The number of approaches by females differed depending on their mating status (GLM-Poisson, $F_{1,80}$ = 4.007, P = 0.045), but not on the male mating status (GLM-Poisson, $F_{1,79}$ = 0.442, P = 0.51): females mated to a SDM exhibited more approaches than virgin females, regardless of the mating status of the males (z = -1.997, P = 0.046). The number of escapes differed between females, but differently depending on the mating status of the male (GLM-Poisson, female mating status: male mating status, $F_{3,78}$ = 2.992, P = 0.029): females mated to a SDM exhibited the same number of escapes from virgin males and SDMs (z = -0.205, P = 0.997), while virgin females exhibited more escapes from SDMs than virgin males (z = 2.624, P = 0.043).

Discussion

We showed that (i) virgin females, mated females, and females mated to a SDM exhibited different behaviors, and that (ii) the mating status of the females influences the behaviors of males. Different behaviors were observed according to the reproductive status of the two sexes, although the majority of the females were of the same mating status at the end of the experiment. As pointed out by Martel and Boivin (2011), mating depends not only on the choice of the individual (true choice), but also on the response of the partner (apparent choice). Accordingly, we showed that the mating pattern in this species can be influenced by the SDM's behavior, which in turn impacts both males' and females' choice. Our results thus demonstrate that SDMs influenced the male responsiveness and female attractiveness.

Sperm-depleted males influence the subsequent behavior of both the females they have mated and virgin females. Females mated to a SDM were more attracted to males than virgin females whatever the reproductive status of the males. Moreover, virgin females exhibited more escapes from SDMs than from virgin males. Taken together, these findings show that (i) females mated to a SDM were more reproductively active than virgin females, and that (ii) SDMs are not as attractive for females as virgin males. Females mated to a SDM should be as least as active as virgin females in order to compensate for the unsuccessful insemination (Arnqvist and Nilsson 2000; Hosken et al. 2001; Chapman et al. 2003; Tregenza et al. 2006). Our results are consistent with this and demonstrate that even if SMDs transferred seminal fluid to the females, this did not inhibit female sexual receptivity as it was demonstrated for other species (Leopold 1976; Avila et al. 2011). Female *A. tabida* mated to a SDM are thus receptive to mating, as has been shown for females *Trichogramma evanescens* mated to a SDM (Damiens and Boivin 2006). Moreover, females mated to a SDM seemed to exhibit adaptive behavior, because they reduced the number of approaches they made toward SDMs; whatever the mechanism behind this post-mating effect, females mated to a SDM behave as if they can "learn" and consequently modify their behavior. This is comparable to other types of behavior exhibited by females in order to ensure that their eggs are fertilized (reviewed by Rhainds 2010).

The behavior of males is also influenced by the reproductive status of the females; virgin females, females mated by a SMD and mated females do not elicit the same behavioral response from males according to the mating status of the males. Our results did not show any significant effect of the reproductive status of the females on the behavior of virgin males (experiment 1). We however noted a tendency of virgin males to exhibit their first courtship and mating toward virgin females, and the small sample size could have led to non-significant results. SDMs, however, did exhibit their first courtship and mating toward virgin females

over females they previously mated. They also exhibited more contacts toward virgin females over females they mated (experiment 2). These results demonstrate that females mated to a SDM are not perceived as virgin females by the males. The preferential choice of SDMs for virgin females rather than females mated to a SDM – even though females mated to a SDM continue to be receptive to mating – suggests a decrease in the attractiveness of females mated to a SDM.

Many cues can be used by males to discriminate between virgin females and females mated to a SDM, and chemicals must play a major role (Thomas 2011). In bumblebees and parasitic wasps, sexual pheromones are indeed known to carry information about mating status (Lloyd 1981; Ode et al. 1995). In *Drosophila*, the cuticular profiles of females change after mating (Steiger et al. 2008). Moreover, the mating behavior of *Asobara tabida* – like many other insect species – is based on a complex and dynamic infochemical emission, and on the perceptions of both males and females (Stökl et al. 2014). *Asobara tabida* males may thus perceive and respond to chemical changes in the mated females (with or without sperm being stored), which enable them to discern the female's reproductive status.

Males could be more attracted to virgin females than to females mated to a SDM because they never encountered the virgin females they faced with (the so-called 'Coolidge effect') (Wilson et al. 1963; Dewsbury 1981). Based on individual marking recognition, Steiner et al. (2008) have shown that males of the burying beetle *Nicrophorus vespilloides* Herbst (Coleoptera: Silphidae) use female cuticular patterns to discriminate between novel females and those they have previously inseminated. In our study, such a "Coolidge effect" may be responsible for the choice of males faced by two mated females (experiment 3), and the choice of a SDM facing virgin females and females mated to a SDM (experiment 1), but not for that of virgin males in the first experiment. Indeed, the virgin males used in our study had never encountered any females. The change in the behavior of the males and females may

therefore involve different mechanisms, and do not depend on sperm transfer.

To conclude, our study showed that the mating process in *A. tabida* depends on the behavior of both sexes, and is not a simple and unidirectional choice of one sex toward the other. *Asobara tabida* males thus actively choose their mates on the basis of mating status, but females can accept or reject the interested males. SDMs and females mated to a SDM behave in different ways towards fertile males, and virgin or mated females, respectively. As other authors have suggested, the modification of male and female behavior as a result of mating could be a way of improving the fitness of SDMs (Damiens and Boivin 2006) by decreasing the likelihood of subsequent mating by other males through sperm competition avoidance. Many hypotheses have been advanced to explain the female mating failure in nature, including a lack of opportunity for mating (Rhainds 2010). The mating behavior of SDMs may contribute to this phenomenon by making females mated to a SDM look like successfully mated females to other males.

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Figure legends

Figure 1: Behaviors of virgin males (a, b), virgin females and females mated to a SDM (c) during the first experiment. (a) Proportion (± 95 % confidence interval) of virgin males exhibiting their first courtship toward (top, n = 13) and/or mated first with (bottom, n = 9) the proposed females. (b) Mean number (± SE) of behavior exhibited by virgin males toward different types of females. (c) Mean number (± SE) of behavior exhibited by females toward virgin males. (ns.: not significant).

Figure 2: Behaviors of SDMs (a, b), virgin females and females mated to the same SDM (c) during the second experiment. (a) Proportion (± 95 % confidence interval) of SDMs exhibiting their first courtship toward (top, n = 11) and/or mated first with (bottom, n = 11) the proposed females. (b) Mean number (± SE) of behavior exhibited by SDMs toward different types of females. (c) Mean number (± SE) of behavior exhibited by females toward the SDM. (ns.: not significant; * $P < 0.05$).

Figure 3: Behaviors of fertile males (a, b), females mated to the same male or to another male (c) during the second experiment. (a) Proportion (± 95 % confidence interval) of fertile males exhibiting their first courtship toward (n = 22) the proposed females. (b) Mean number (± SE) of behavior exhibited by the SDM toward different types of females. (c) Mean number (± SE) of behavior exhibited by females toward the SDM. (ns. not significant; ** $P < 0.01$).

Figures

Figure 1

Les paramètres nécessaires sont manquants ou erronés.

422 Figure 2

423 **Les paramètres nécessaires sont manquants ou erronés.**

424

Figure 3

Les paramètres nécessaires sont manquants ou erronés.